STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

THE CERTIFICATION OF STAINS (*Continued*)

In the last two numbers of this journal[1] there has been inserted a series of notes on the staining problems that have occurred in the standardization of certain biological stains. The dyes so far discussed in this way have been brilliant cresyl blue, brilliant green, the eosins, erythrosin, and basic and acid fuchsin. The discussion is now continued with notes on a few other dyes that are now on the certification basis.

GENTIAN VIOLET

(*Including crystal and methyl violets*)

The name gentian violet does not occur in any dye index, as no dye of this name is recognized in the dye industry at the present time. Apparently, when microscopists first began to use dyes in their staining procedures they obtained under the name of gentian violet one of the dye mixtures now most commonly known by the name methyl violet. This dye proved sufficiently useful so that later, after the chief source of biological stains had become Dr. G. Grübler and Company, this concern continued to furnish biologists with a dye of this designation, even after the name had become obsolete in the dye industry. The statement usually accepted by biologists as to the nature of gentian violet was that it was a mixture of crystal violet and methyl violet, together with a certain amount of dextrin.

When biologists began to study the chemistry of dyes the indefinite nature of this definition was quickly realized. This arose from the fact that methyl violet itself is a mixture of dyes, one of which is ordinarily crystal violet. Crystal violet is hexamethyl pararosanilin, while the methyl violets are mixtures of this compound with varying proportions of the pentamethyl and tetramethyl derivatives of the same compound. As the hexamethyl derivative is the bluest of the

[1]Conn, H. J. Progress in the standardization of stains. The certification of stains. Stain Techn., **9**, 113–5. 1934.

———— Progress in the standardization of stains. The certification of stains. Stain Techn., **10**, 3–5. 1935.

series, it becomes evident that the methyl violets vary in shade from a reddish to a bluish violet, according to the extent of their methylation. The different shades are ordinarily loosely noted in the trade by such designations as R, B, 2B, 4B, etc. It is evident, accordingly, that adding more crystal violet to any methyl violet merely deepens the shade of the dye without adding any ingredient that was not already present in the methyl violet before mixing with the crystal violet.

This situation was undoubtedly appreciated by the stain companies in the United States at the time when imported stains were no longer available; but when the Stain Commission began investigating gentian violet it was found that each producer was interpreting gentian violet in a different way and no two of the products on the market under this name were alike. Everything from crystal violet to a fairly red methyl violet was sold under this designation, sometimes fairly pure and sometimes mixed with as much as 50% dextrin.

Accordingly, one of the first problems facing the Stain Commission was to define gentian violet. Before doing this an investigation was necessary to determine which of these dyes was desired by biologists for the purpose for which gentian violet was ordinarily used. Part of this investigation had already been carried on under the auspices of the Society of American Bacteriologists,[2] but still further investigation by the Stain Commission was necessary before definite conclusions were reached.[3]

The first point that had to be determined was which of the various uses of gentian violet were the most critical. It was evident, for instance, that when the dye was used for simple direct staining without a counterstain, almost any dye of this group might well give satisfactory results, but in the most complicated procedures more exact requirements might have to be met. The two procedures that were finally selected as presenting the most critical demands in the matter of gentian violet were the Gram stain for bacteria and the Flemming triple stain for cytological preparations. The investigation of these points soon brought out two or three facts of significance.

In the first place, it became evident that the dextrin often present in gentian violet played no active part in either staining procedure, but acted merely as a diluent for the dye. In the second place, it was apparent that the behavior of the various members of this group of dyes in the two staining technics was practically

[2]Conn, H. J., *et al.* An investigation of American gentian violets. Report of Committee on Bact. Technic of Soc. Amer. Bacteriologists. J. Bact., **7**, 529–36. 1922.

[3]Conn, H. J. Progress in the standardization of stains. Grades of gentian violet. Stain Techn., **1**, 53–5. 1926.

the same, the only difference in results being due to the difference in shade of the individual compounds. Shade, however, proved to be a matter of considerable importance because of the necessity of contrast with the other dyes used in these staining procedures. It was found that ordinarily the bluer members of the group gave the best results. For this reason, one could employ with greatest satisfaction either crystal violet or one of the very blue gentian violets. Accordingly, the Stain Commission finally adopted the following as a definition of gentian violet:

> Gentian violet for general staining purposes, as defined by the Commission on Standardization of Biological Stains, must be either pentamethyl or hexamethyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B. In other words, the dye must be Colour Index No. 680 or No. 681.

This definition practially allows the sale of a crystal violet or of one of the most highly methylated methyl violets under the name of gentian violet. The Stain Commission, however, recommends the purchase of crystal violet, as such, for the procedures for which gentian violet is called; but the adoption of the above definition makes it possible for stain companies to meet the demand for a dye of this name, a demand which will probably continue for some time yet.

Beyond this there has been no real problem with the standardization of gentian violet. Crystal violet is very constant and practically all that is put on the market meets the biologists' requirements. As a matter of fact, a very large portion of the gentian violet now sold in the United States is really crystal violet. To avoid confusion biologists are urged to order it as crystal rather than gentian violet.

HEMATOXYLIN AND HEMATEIN

Both hematoxylin and its oxidation product hematein are, as is well known, natural dyes derived from logwood. Altho not synthetic dyes, their standardization has been pretty well effected, thanks to the fact that there is only one firm in America which manufactures them, altho the product of this manufacturer is handled by all the regular dealers in biological stains.

When hematoxylin of American manufacture was first put on the market it was not sufficiently purified and, accordingly, of a dark color. On account of the complaints due to this color the manufacturers began bleaching it as well as purifying it in an effort to obtain a more satisfactory stain. Investigations soon showed, however, that the bleaching process was somewhat harmful and it has since been omitted. The product which has now been on the market

for a good number of years is purified to a greater extent than the earlier product but as no bleaching process is carried out the color is darker than some of the first American hematoxylin that was available. No serious complaints in regard to hematoxylin have been received by the Stain Commission for a good many years and it is, therefore, regarded as standardized in a practical way, even tho it is not a definitely known chemical compound and its scientific standardization may still be out of the question.

METHYLENE BLUE

In the early days of stain standardization some difficulties were encountered in connection with methylene blue. These difficulties were in a way unnecessary and had been introduced largely because there were then two forms of methylene blue on the market, one a simple methylene blue chloride and the other a double salt, the chloride of zinc and methylene blue. This latter was cheaper and for that reason ordinarily employed in the textile industry, while the former being less toxic and more expensive had come into use almost wholly for medicinal purposes. Both salts were furnished to biologists as stains and it was recognized that for some purposes (notably in blood stains) medicinal methylene blue was best. The zinc double salt, however, well diluted with dextrin, was furnished under the label: "methylene blue, for bacilli". It was naturally assumed from this designation that this was a better stain for bacteria than the medicinal methylene blue. It happens, however, that one of the most common formulae for a bacterial stain calls for a certain proportion of a saturated alcoholic solution of the dye, and the zinc double salt is practically insoluble in alcohol. The fact caused much confusion.

One of the first investigations of the Stain Commission, however, showed that there is no staining procedure for bacteria or for histological preparations for which the zinc double salt is better than methylene blue chloride. Accordingly, stain companies have been asked to furnish the latter salt for all biological purposes and since doing so no further problems in connection with this dye have arisen.

—H. J. Conn.

(To be continued)

MARCHI'S STAINING METHOD: III. ARTEFACTS AND EFFECTS OF PERFUSION

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ABSTRACT.—**The following classes of artefacts in preparations made by the Marchi staining method have been recognized and investigated: Intrinsic artefacts, traumatic artefacts, perfusion artefacts and artefacts produced by ether anesthesia. The character of the artefacts and the method of their production indicates that they are physically changed myelin which stains as a result of an alteration in its structure.**

An experiment was also carried on to indicate the effect on the stain preparations of perfusing the animal with various fluids. It was found that certain fluids (formalin, 10%; Mueller's fluid; and NaCl, 10%) caused an increase in dust and an edema of the brain, while others (Mueller's fluid plus 10% $MgSO_4$; and $KClO_3$, 5%) caused a decrease.

SOURCES OF ARTEFACTS

Intrinsic artefacts. In a previous paper[2] attention was called to a type of interstitial granular deposits, termed dust, which occurred to a varying degree in all Marchi preparations. Another type of artefact apparently present only after long formalin fixation was also described. Since then a series of brain stems and spinal cords (rabbit and cat) have been examined. It was found that the dust varies considerably in different parts of the same spinal cord and brain (mesencephalon, pons, and medulla oblongata) as well as individually among animals. Granular precipitation, however, was more abundant in areas occupied by myelinated fibers (white matter of the spinal cord and pyramids of the medulla oblongata) than in nuclear regions (gray matter of the spinal cord and the tegmentum of the medulla oblongata). In specimens where the dust was most abundant the ganglion cells also contained numerous small black globules.

Traumatic artefacts. Pulling a small thread thru the center of a block of tissue following formalin fixation caused the immediately surrounding tissue to stain black (Fig. 1). This phenomenon does

[1]Contribution No. 212.

[2]Swank, R. L. and Davenport, H. A. Marchi's staining method: II. Fixation. Stain Techn., 9, 129. 1934.

not conform with the accepted idea that traumatic artefacts are producible before fixation, but not afterwards.

To demonstrate and study post-fixation artefacts, four blocks of tissue (each from a different animal) were pinched with forceps after formalin fixation, and stained by the Marchi's fluid plus 2% acetic acid. Each of the blocks presented an area of diffuse staining opposite the point where the forceps had been applied. The next step was to find out the amount of pressure necessary to produce these artefacts and under what condition they occur in both fresh and formalin fixed tissues.

Four blocks of unfixed spinal cord were subjected to varying degrees of pressure. The first, intended as a control, was not squeezed; the second was squeezed lightly; the third moderately; and the fourth was crushed. Also four formalin fixed blocks from the same animal were subjected to similar degrees of pressure with the exception of the maximum grade which crushed the tissue. The first four blocks of traumatized fresh tissue were then fixed in 10% formalin and all were stained (without washing) in Marchi's solution plus 2% acetic acid.

Examination of the sections revealed evidence of trauma (Figs. 2 and 3) in all blocks in which the pressure had been sufficient to rupture the tissue or fragment the myelin. Two of the unfixed (light and moderate pressures) and one of the fixed (light pressure) showed no traumatic staining artefacts even tho distortion of the block had been produced, but closer examination of the sections revealed that actual damage to the tissue had not occurred. In the remaining blocks, marked diffuse granular staining was present at the site of the injury, and it was evident that either fragmentation of the myelin or its displacement (with or without tearing of the neuroglia) had occurred.

Figure 2 demonstrates that traumatic changes are not limited to the area of the lesion. Many fascicles containing degenerating globules of myelin could be followed from the lesion for a distance of 5 to 6 mm. Such degeneration arising from trauma insufficient to tear the neuroglia (Fig. 3) would be very confusing, as evidence for its cause would not be present.

To determine if peripheral myelinated nerves behave in a similar manner, normal sciatic nerves from two cats were subjected to the following procedure. One piece of nerve from each cat was used as a control; a second was stretched before fixation; a similar tension was exerted on a third after fixation; and a fourth was pinched after fixation. The fresh, traumatized nerves were then fixed in formalin, and all were stained. Traumatic artefacts were present in the nerves

stretched before fixation, but not in those subjected to similar tension after fixation. Displacement of the myelin to the interstitial spaces had not occurred, but occasional fragmentation of the myelin within the sheaths, which resulted in black globular staining, was present. Many normal appearing myelin rings stained gray to black (Fig. 4), indicating that alterations in the physical state of the myelin insufficient to cause fragmentation had occurred also. In the nerves which were squeezed after fixation artefacts were present in the interstitial spaces and many neurilemmal sheaths contained fragmented myelin, both of which stained black (Fig. 5). Apparently fragmentation of the myelin, with or without displacement, results in black staining, and a gray (often black) staining of non-fragmented myelin results from trauma to peripheral nerves. This experiment shows that greater trauma is required to produce artefacts in peripheral nerves than in the central nervous system, for very severe trauma was necessary to produce artefacts in peripheral nerves.

The greater difficulty encountered in producing traumatic artefacts in peripheral nerves seems to be due to the presence therein of neurilemmal sheaths. Apparently the neurilemmal sheaths prevent the myelin from being fragmented or displaced except by the severest trauma. In the central nervous system a complete encasement for the myelin is lacking. Here the neuroglia form a fenestrated support from which the myelin can be squeezed readily. Fresh brain and spinal cord is very pliable and readily molds itself to applied pressure, hence, tearing of the tissue is difficult to produce, the neuroglial framework stretches to accommodate itself to the pressure, and myelin molds itself to the changed shape of its supporting tissue. On the other hand, formalin fixed brains and spinal cords are very friable and break or tear rather than mold themselves to applied instruments, hence, the myelin is fragmented or forced into the interstitial spaces.

The staining which occurs in normal peripheral nerves as they leave the anterior gray column, or in the oculomotor or other normal cranial nerves is often seen to be globular and stained black, demonstrating a fragmentation of the myelin (Fig. 7). More often these nerves appear to be non-fragmented and stained gray to black. Because of their positions, abnormally great tension is often exerted upon them during removal of the brain and spinal cord. This produces alterations of the physical form of the myelin so that it stains. The degree of the physical changes produced in the nerves probably determines whether they will stain black or merely gray. As artefacts are produced with difficulty in fixed peripheral nerves, fixation

of these nerves *in situ* appears to be the procedure of choice. Conversely, the central nervous system should be fixed after its removal from the animal.

Perfusion artefacts. Formalin perfusion is often employed as a method for fixing tissues of the central nervous system which are to be stained by the Marchi procedure. To test the effect of perfusion, two cats were perfused with warm (37°C.) normal saline solution followed by perfusion with 10% formalin. Shortly after, the brains and spinal cords were removed and placed in formalin for 48 hours. As the brains and spinal cords were tightly swollen within the cranium and vertebral canal, and were very friable, no amount of care during their removal could prevent the tissues from being frequently traumatized sufficiently to produce staining artefacts. Microscopic examination moreover, revealed a marked increase in interstitial granular deposits very similar in appearance to the intrinsic artefacts found in non-perfused animals. The tissues from numerous perfused animals have been examined since then, and all have been characterized by an abundance of dust far exceeding non-perfused specimens.

Artefacts produced by ether anesthesia. In two guinea pigs killed by long etherization (2 hours) there was a marked increase in granular precipitation (Fig. 6). Weil[3] described histological alterations of the myelin and the ganglion cells as a result of prolonged etherization.

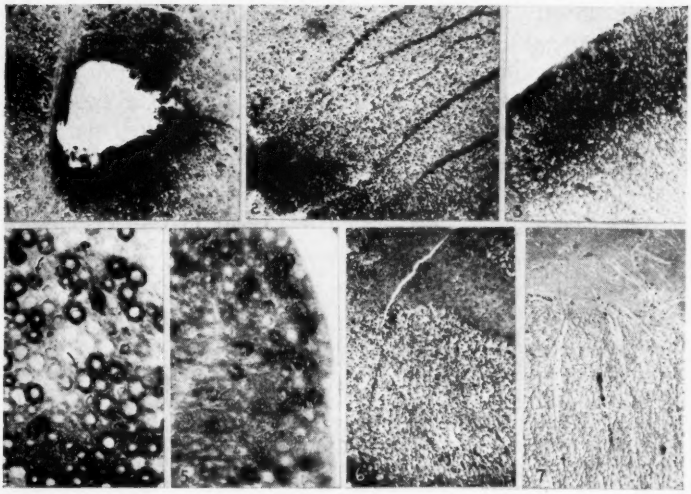
Summary. The character of the artefacts and the methods of their production indicates that they are physically changed myelin (non-fragmented, fragmented, or displaced) which stains as a result of an alteration in its structure. Most of the artefacts are interstitial, but the non-fragmented and fragmented types present inside the neurilemmal sheaths of peripheral nerves appear to be due to an alteration without an interstitial displacement of the myelin.

Examination of Marchi preparations following various periods of degeneration in the cat show that the earliest staining of degenerating myelinated sheaths occurs four days after removal of their trophic cell bodies. It is at this time that the first fragmentation of the myelin occurs.[4]

By the end of eight or nine days discrete globules have appeared and increased to a maximum number between 14 and 20 days. Probably the cause of staining artefacts (interstitial or interneurilemmal)

[3]Weil, Arthur. Veränderungen in dem histologischen Bilde und dem Lipoidaufbau des Zentralnervensystems in der Äthernarkose. Arch. Gesamte Physiol., **223,** 351. 1929.

[4]Ranson, S. W. Degeneration and regeneration of nerve fibers. J. Comp. Neur., **22,** 487. 1912.



EXPLANATION OF FIGURES

All figures × 200. All tissues illustrated here were fixed in formalin after their removal from the animal and were stained without washing in Marchi's solution plus 2% acetic acid.

Figure 1. Photomicrograph of a section of the medulla oblongata of a cat showing the area thru which a silk thread had been drawn after fixation of the tissue in formalin.

Figure 2. Photomicrograph of a section thru the mesencephalon of a cat. In the lower left corner is an area of diffuse staining which was caused by traumatizing tissues after fixation. Note the small fascicles containing stained globules of myelin which appear to arise from this area and look like degeneration streaming toward the upper right corner of the photograph.

Figure 3. Photomicrograph of a section of the spinal cord of a cat. The diffuse staining was the result of post-fixation trauma. Note that the neuroglial tissue appears undamaged; the myelin was squeezed into the interstitial spaces.

Figure 4. Photomicrograph of a section of the sciatic nerve of a cat. This nerve was stretched before fixation. Note the numerous black and gray interneurilemmal myelin rings and the occasional fragmented ring. There is very little evidence of interstitial granular precipitation in the section.

Figure 5. Photomicrograph of a section of the sciatic nerve of a cat. This nerve was squeezed with forceps after fixation. Note the fragmented interneurilemmal myelin sheaths and the interstitial granular precipitation.

Figure 6. Photomicrograph of a section of the spinal cord of a guinea pig This animal was killed after 2 hours etherization. Note the abundance of dust.

Figure 7. Photomicrograph of a section of the spinal cord of a cat. This shows a motor nerve soon after leaving the ventral gray column containing fragmented myelin, similar to that which was produced in control preparations by trauma.

is predominantly physical in nature. Chemical factors cannot be ignored but appear to be of secondary importance. Physical changes in the myelin during Wallerian degeneration appear concomitant with its selective staining. Whether this is the cause of staining was not determined, but it seems significant that the production of physical changes in a normal nerve by trauma results in similar staining.

PERFUSION WITH VARIOUS ELECTROLYTES

Nine previously decorticated cats were utilized in this experiment. Two non-perfused brains and spinal cords which were fixed in formalin previous to staining served as controls. The remaining seven animals were perfused with the various solutions indicated in the table. Following perfusion, the brains and spinal cords were removed to formalin (or some other fixative indicated in the table) for 48 hours before staining in Marchi's solution plus 2% acetic acid. Also blocks from each animal were washed before staining, and a few blocks (non-perfused) were fixed directly in the fixatives listed in the table. Reference to these will appear in the discussion.

In the table are recorded: the treatment of the tissues; gross changes produced in the brain by perfusion; the amount of dust present in the preparation; and the quality with which both the normal and degenerating myelin stained.

The idea behind the use of various perfusing solutions was suggested by the observation that perfusion with formalin caused an edema (swelling) of the brain with increase of dust. It was thought that transudation of fluid from the blood vessels to the interstitial spaces or softening and migration of myelin might account for increased granular precipitation. Consequently, perfusion with hypertonic solutions was tried on the theory that dehydration of the tissues would result in a reduction of dust.

Reference to the table shows that some fluids caused an increase in dust (formalin 10%; Mueller's fluid; and NaCl 10%) whereas others caused a decrease (Mueller's fluid plus $MgSO_4$ 10%, and $KClO_3$ 5%). In the first group marked edema of the brain (indicated by its swollen condition) was present, while in the second group edema was absent. The brain perfused with $KClO_3$ (exp. 5) was edematous, but apparently the $KClO_3$ inhibited staining of the interstitial granular deposits. Stated roughly, the amount of interstitial granular precipitation was proportional to the degree of edema of the brain. The absence of edema (and dust) in experiment 6 can be attributed entirely to the presence of hypertonic $MgSO_4$, as Mueller's fluid alone caused an increase in dust.

TABLE 1

EFFECT OF VARIOUS PERFUSION FLUIDS ON MARCHI'S STAINING

Exp.	Perfused with	Removed to	Gross changes of C.N.S.	Dust	Staining of normal myelin	Degeneration
1	NaCl 10%	formalin 10%	very soft and edematous, slightly swollen	++++	few scattered; stained lightly	poorly stained
2	Mueller's fluid	formalin 10%	soft, slightly swollen	++	few scattered; stained lightly	well stained
3[5]	formalin 10% (2 animals)	formalin 10%	very firm and swollen tightly against cranial vault	++	few scattered; stained lightly	well stained
4	control (not perfused) (2 animals)	formalin 10%			none	well stained
5	$KClO_3$ 5%	formalin 10% + $KClO_3$ 5%	very firm and swollen tightly against cranial vault	—	none	well stained
6	Mueller's fluid + $MgSO_4$ 10%	formalin 10%	soft and decreased in size	——	large number darkly stained	well stained
7[5]	$FeCl_3$ 10%	formalin 10% + $FeCl_3$ 10%	very hard, and extremely shrunken	——	very large number; stained darkly	indistinct as too many normal rings are stained

The stained, normal myelin rings seen in experiment 6 and 7 can be attributed to $MgSO_4$ and $FeCl_3$, respectively. They seem to act in a manner very similar to an acidified fixative.

In all preparations excepting those from experiments 1 and 7 the degeneration was well stained. As reported previously,[6] washing the tissues before staining removed most of the dust, and at the same time a noticeable portion of the degeneration.

[5]These animals were perfused with normal saline solution after which they were perfused with the solutions listed in the table.

[6]Swank, R. L. and Davenport, H. A. Marchi's staining method: Studies of some of the underlying mechanisms involved. Stain Techn., 9, 11. 1934.

Perfusion with hypertonic sodium chloride solution (10%) caused edema of the brain and a great increase in dust (exp. 1). Chambers and Reznikoff,[7] while studying the effects of the cations Mg, Ca, K, and Na upon the protoplasm of *Ameba proteus*, found that whereas NaCl weakens and disrupts the surface membrane of the ameba, the chlorides of K, Mg, and Ca have no such effect. Furthermore, NaCl increases the fluidity of and $MgCl_2$ solidified the internal protoplasm of the ameba. These facts gather increased significance when it is recalled that the cell wall is often considered to be a phospholipin similar to myelin. Probably disruption of the myelin occurred in the specimen perfused with 10% NaCl while its stability was increased by perfusion with $MgSO_4$.

[7]Chambers, R. and Reznikoff, D. Micrurgical studies in cell physiology. I. The action of the chlorides of Na, K, Ca, and Mg on the protoplasm of ameba proteus. J. Gen Physiol., **8**, 369. 1926.

NEGATIVE STAINS IN THE DEMONSTRATION OF BACTERIA

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ABSTRACT.—**Since certain objectionable features have been observed in the old technics of preparing negatively stained bacteria slides, modifications of these methods have been attempted, and studies have been made of various dyes not heretofore described as negative stains. India ink, nigrosin, indulin, Congo red, Poirier's blue, anilin blue (methyl blue), China blue, blue de Lyon, blue de Lyon O, and night blue are recommended. Dyes of the same name, but sold by different companies, often present different effects.**

For the demonstration of bacteria in high school and college courses in biology, the negative staining technics have been found to be exceptionally valuable. Their advantages over the better established positive procedures may be stated as follows: negative methods are simple and rapid; the bacteria are more strikingly presented; the organisms are not previously treated by heat or mordants, and are, therefore, observed in a more nearly natural state; films so prepared are fairly permanent; the bacteria may be seen clearly under a 4 mm. objective without the aid of oil immersion. One of the larger biological supply houses,[1] recognizing these advantages, now features sets of slides for demonstration purposes, in which the negative preparations have been used almost exclusively.

A considerable bibliography on negative staining methods has been compiled by other authors.[2]

I have recently had occasion to prepare a number of negatively stained slides of bacteria and have noted in some of the early technics certain objectionable features, which have led me to make a comparison of these older methods and to test a number of stains

[1]The Central Scientific Company, Chicago, Illinois.

[2]Dorner, W. Ch. The negative staining of bacteria. Stain Techn., **5**, 25. 1930. (Adequate discussion is here given of the India ink and nigrosin methods.)

Lehmann, K. B., and Neumann, R. O. Bakteriologie, Band I, pp. 5 and 13. Lehmanns, Munich. (Various modifications of the India ink technic are considered.)

Maneval, W. E. Rapid staining methods. Science, **80**, 292–4. 1934. (Modifications of the nigrosin technic by the addition of other dyes are well discussed. The Benian Congo red method is also considered.)

Meyer, K. F., et al. Practical Bacteriology, Medical Zoology, and Immunology, 386. 1925. University of California Press. (Eisenberg's "Cyanochin" method, as well as other negative methods, are adequately treated.)

which do not appear to have been previously used as background stains. India ink, as is well known, presents a granular background that is often confusing. The ink is sensitive to the acidity of the culture; and in the presence of fat globules, as are often encountered in milk cultures for example, difficulties in staining frequently arise. With the Congo red staining technic the color often changes on the slide from the blue acid to the red alkaline state, or it may fade out altogether. Furthermore, cracks in the film often occur, even tho meticulous care be taken to prevent them.

Three brands of nigrosin have been compared, the Fischer technic having been employed in making the comparisons. The products used were: nigrosin, Grübler; nigrosin water soluble, Coleman and Bell, (stamped "2213"); nigrosin water soluble, National Aniline and Chemical Co., (Shultz No. 700, C. I. No. 865) Cert. No. NNi-4. Altho the color of the dyes varied from a purple to a black, the products of all three companies gave excellent results when 5% aqueous solutions were used. It was found that the cracking of the film on the slide could be prevented by spreading a drop of the dye well over the whole surface of the slide in which the bacterial material had been mixed, and then tilting at an angle of from thirty to sixty degrees so that the excess stain could drain off.

A possible differential use of nigrosin in combination with certain other dyes such as rose bengal, Magdala red, etc., has recently been reported by Maneval. (See footnote 2.)

Indulin has been found to give results fully comparable to nigrosin. The following brands were tested: indulin, Grübler; indulin (bluish), National Aniline and Chemical Co., (Schultz No. 697, Lot No. 3731); indulin water soluble, Coleman and Bell Co., (stamped "2214"). The colors varied, some of the indulins appearing much like the nigrosins. The technic is the same as the Fischer nigrosin technic and has the same advantages. Five per cent aqueous solutions of any of the brands of indulin were found to give excellent results.

Eisenberg's "Cyanochin" method has been compared with the other methods, and it was found that a 5% to 10% aqueous solution of China blue (Coleman and Bell) with 1% cyanosin (Coleman and Bell) produced an attractive background stain. The technic is simple, and the results are good. Cracks often appear in the film, but they may be minimized by tilting the slide while the film is drying.

Six dyes have been found to be exceptionally good in producing blue backgrounds. Listed by label, in the order of their desirability, they are:

Poirier's blue (cotton blue), National Aniline and Chemical Co., (Lot No. 5387).

Anilin Blue (methyl blue), Coleman and Bell, (Cert. No. CK-2).

China blue, Coleman and Bell.

Blue de Lyon, Grübler.

Blue de Lyon O, Coleman and Bell.

Night blue, Coleman and Bell.

The first five of these dyes may be prepared in 5% aqueous solutions, while the night blue should be prepared in saturated solution. All of them should be heated to get all the dye in solution, and then filtered several times thru the same filter paper. Methods of application are the same as in the nigrosin technic. The night blue is irregular in its activity, as it sometimes stains the organisms and at other times obscures them.

Certain other dyes have been found to be generally inferior to the dyes just listed, altho they could be used if it became necessary. These stains, listed in the order of their utility, are:

1. Anilin blue water soluble, National Aniline and Chemical Co., (Schultz No. 539, C. I. No. 707) Cert. No. NK-1.
2. Lyons blue, Coleman and Bell.
3. Water blue, Coleman and Bell.
4. Soluble blue, Coleman and Bell.
5. Methyl blue, Coleman and Bell.

Five per cent aqueous solutions of these dyes seem to be most nearly desirable. Methods of application and preparation are the same as for the other blue dyes.

Either because of a lack of homogeneity and denseness, or because the bacteria were stained, or because many cracks were formed in the films, the following dyes were found unsatisfactory as background stains:

1. Wool black, Coleman and Bell.
2. Anilin blue black, Coleman and Bell.
3. Anilin blue black, National Aniline and Chemical Co., (Lot No. 2025).
4. Bleu Lumiere, Coleman and Bell.
5. Methyl blue, National Aniline and Chemical Co., (stamped "3421").
6. Black NBR, Coleman and Bell.
7. Berlin blue, Coleman and Bell.

Dr. H. J. Conn[3] has pointed out that practically all of the blue dyes herein considered are very similar and many of them supposed

[3]Personal communication.

to be identical. With the exception of Lyons blue, methyl blue, and night blue, these dyes correspond to C. I. No. 707. Methyl blue is generally accepted as No. 706, while the other two names are applied to No. 689. The dye with C. I. No. 689 is a basic dye; and No. 706 and No. 707 are acid dyes derived from it by sulfonation. Since the method of manufacture yields varying degrees of phenylation and sulfonation, the Colour Index number in each instance really refers to a group of dyes rather than to single compounds. The process is so difficult to control that the manufacturers make little claim as to the possibility of duplicating any batch that has proven satisfactory for any special purpose. This possibly explains why the products of some manufacturers were found good and some poor for background staining. It also suggests that one cannot depend on another lot of one of these dyes to give the same results as those herein indicated.

TECHNIC FOR STUDYING CHROMOSOME STRUCTURE

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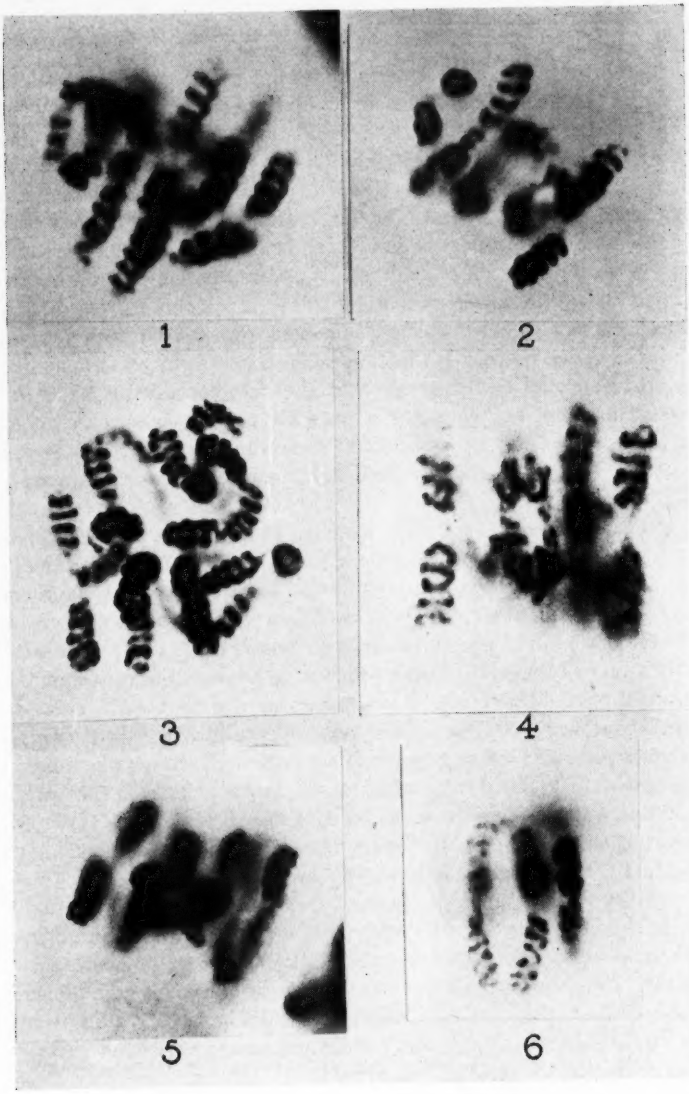
Abstract.—**The methods described are modifications of various technics for the study of spiral structure in chromosomes. They enable permanent preparations to be made with better fixation and allow the use of stains which give clear and more critical definition. The first method described involves the use of ammonium hydroxide (880 vols.) fumes for the treatment of pollen mother cells before fixation. Anthers of Tradescantia are smeared on a slide and wet in a 3% cane sugar solution. The preparation is then immediately placed in a dish of fixative where it remains for two hours. The slide can then be washed, bleached and stained with gentian violet or hematoxylin. It was found that fumes of nitric acid, hydrochloric acid and glacial acetic acid gave similar results. For the second method, boiling water is used for pre-treatment. A smear is made on a slide and immersed in boiling water for five to ten seconds. The smear is then fixed and treated in the usual manner.**

Recent advances in cytology have led to the need of new methods in technic, especially for the demonstration of spiral structure in chromosomes. Several methods have been devised, some more successful than others. Nebel (1932) has demonstrated the effects of dessication and special fixatives. Sakamura (1927) published photographs of unstained cells which were killed with hot water, the coiled-thread nature of the chromosomes being quite clear. Kuwada and Nakamura (1934) have been very successful with cells of *Tradescantia reflexa* treated with ammonia fumes and afterwards fixed in aceto-carmin. The disadvantage of the latter method is that preparations when made permanent by McClintock's method (1929) are never as clear as the original semi-permanent slides. This is due to the fact that the ammonia has a preliminary fixing effect which prevents very deep staining. These lightly stained cells are actually better for study, as the spirals are more drawn out when a large dose of ammonia is used. It seemed that a method for making permanent smears, substituting an osmic fixative for aceto-carmin and allowing the use of better stains such as gentian violet or hematoxylin, would be useful. It was found that preparations were somewhat superior when made by the methods described below.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Methods

The only materials available for trial were pollen mother cells of plants of *Tradescantia rubra* and *T. virginiana*, both tetraploids. The anthers of a bud at the correct stage of division are smeared on a clean slide. The slide is immediately immersed, cell side down, for a second or two in a dish containing 3% cane sugar solution. The preparation is then laid flat for a few seconds over a jar containing concentrated (880 volumes) ammonium hydroxide. A rectangular staining jar 3½" (high) x 2½" x 1¼" was used, but 3" x 2" x ¾" would be better. The slide is then placed horizontally in a dish of fixative. Medium Flemming's solution gives slightly better preparations than Navashin's or 2 BE. Fixation is complete after two hours. The smear is then washed, bleached and stained in the usual manner.

It was found that ammonia could be replaced by acids such as concentrated nitric and hydrochloric and glacial acetic acid. The results obtained are similar, with a slight preference for nitric acid. In the best slides, coiling can be seen in 50 to 80% of the cells, but the result is always variable. Fixation is very good in that the structure is shown clearly and consistently and the cytoplasm is not shrunken. The results obtained depend on ones ability to keep the smear on the slide. The preparation must be immersed flat in the sugar solution and removed horizontally; if the slide is canted to one side, many more cells are washed away. It is possible that plants with very juicy anthers will not require wetting in sugar solution.

Experiments were also tried with boiling water. The anthers are smeared on a slide and immediately placed cell-side down in a dish containing boiling water; five to ten seconds will suffice. The smear is then quickly inverted into a dish containing fixative. A longer time in hot water tends to spread the chromosomes and stretch out the spirals. Fixation and after-treatment is normal. The same care

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Explanation of Plate

Figures 1–4. Photomicrographs of pollen mother cells of *Tradescantia rubra*.

Figures 5 and 6. Photomicrographs of pollen mother cells of *Tradescantia virginiana*.

Cells treated before fixation in medium Flemming.

1. Metaphase I. Ammonium hydroxide.
2. Metaphase I. Glacial acetic acid.
3. Diakinesis. Nitric acid.
4. Metaphase I. Hydrochloric acid.

5 and 6. Metaphase I, boiling water; 5 shows fragments pairing.

should be taken with this method when the slide is put in and taken out of the hot water. The best slides are equal to those obtained with the fume technic. These technics were not successful with pollen grains; it is probable that the thickened pollen grain wall prevents the treatment from being effective. Finally the effects of steam were tried with some success, but lack of material prevented a thoro trial. This method would possibly reduce the risk of losing cells.

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STAINING THE SKELETON OF CLEARED EMBRYOS WITH ALIZARIN RED S

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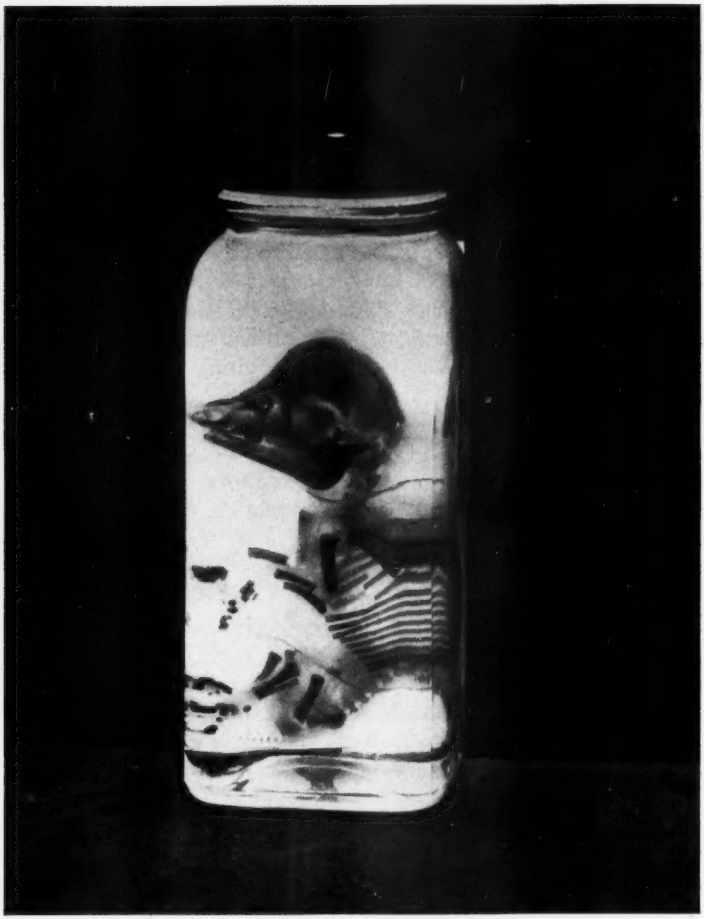
Alizarin red S offers a selective, progressive method for the staining of skeletons of cleared specimens. The stain, when used under the proper conditions, is specific for bone. Dawson (1926) used 95% alcohol as the fixing medium. A later worker, Miss Ignalzi (1929) used 10% formalin for this purpose. I have found that the specimen will clear faster and with less subsequent maceration if fixed in alcohol rather than in formalin. Fixation requires at least 48 to 96 hours, depending upon the size of the specimen. The longer fixation is more desirable, for then there will be less maceration occurring in the KOH that follows. After fixation the specimen is placed in a 2% solution of KOH until the bones are clearly visible thru the jelly-like body tissue. The success of the whole process depends upon the proper degree of clearing in this solution of KOH, for if the specimen be insufficiently cleared, a general staining of all the body tissues will occur. After clearing, the specimen is transferred to a dilute solution of alizarin red S in KOH (1 part of alizarin to 10,000 parts of 2% KOH). The stain is allowed to act until the desired intensity of color in the developing bone is attained. Twenty-four hours is usually sufficient. After staining, the clearing process is completed in increasing concentrations of glycerin. The specimen is stored in pure glycerin. (See plate on p. 62.)

Should the tissue be excessively stained, the specimen may be decolorized by placing in acid alcohol (1% sulfuric acid in 95% alcohol). This method has its faults for complete decolorization may occur, and the minute ossification centers present may decalcify. The specimen may also be allowed to stand in strong sunlight or in ultra-violet light which will remove the stain from the tissue surrounding the bone. In this case, the bone will also be slightly decolorized.

Rat, pig, human, bovine, cat, and chick embryos were stained by this method. The rat, pig, and cat stained most successfully. Clearing chicks in KOH is difficult, probably due to some factor in the skin. Work towards the elimination of this difficulty is now in progress.



A Seven Centimeter Pig Embryo Stained with Alizarin Red S

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LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked.

An Application of Blood Staining to Formalin Fixed Tissues

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Reprinted from J. Techn. Methods & Bull. Int. Assoc. Med. Mus., **14**, 68. 1935.

Polychrome staining of formalin fixed tissues presents a technical problem that has been solved, more or less satisfactorily, in a variety of ways. No claim to originality is made for the following combination of recognized methods. We offer it because, in our experience, it gives greater differentiation and precision of staining than previous adaptations of the Romanowsky blood stains to paraffin sections of formalin fixed tissues.

1. Xylol.
2. Absolute methyl alcohol, 2 changes.
3. Cover slide with a freshly filtered saturated solution of Jenner's stain (National Aniline and Chemical Co.) in methyl alcohol, 3 minutes.
4. Add an equal amount of distilled water—1 minute.
5. Plunge, without washing, into a Coplin jar of dilute Giemsa solution (1 drop of Giemsa's Spirochete Stain from Hynson, Westcott and Dunning, to 1 cc. of distilled water)—45 minutes.
6. Rinse and differentiate in acidulated distilled water (1 drop of glacial acetic acid to 5 cc. of distilled water).
7. Rinse in pure distilled water.
8. Dehydrate quickly in 95% alcohol and two changes of absolute alcohol.
9. Clear in xylol and mount in Canada balsam.

Benavides, J. Comments and procedure on thick blood film technic. J. Lab. & Clin. Med., **20**, 289–95. 1934.

For mass survey work the thick-film method of blood examination fills an important need. Of outstanding value in the tropics, it is intended for the diagnosis of parasites in mammalian blood, rather than for the study of their characteristics or relationship to red blood cells. The author relates in detail the findings of long experience in the laboratory and on the field with the method, covering 200,000 thick films in the last four years. The subject matter discussed includes: slides, equipment, field case, animal survey, source of blood, and staining.

The Giemsa stain in powder form is preferred, using 2.4 g. of Azure-II-eosin (Grübler) in 200 cc. of anhydrous glycerin. The solution is kept 30 minutes at 60° C. and shaken once or twice; 200 cc. of methyl alcohol are added and the heat treatment repeated. After 12 hours at 37° C. the solution is filtered. Equal parts of alcohol and glycerin are used here to insure a more thoro laking of the film than when a larger proportion of alcohol is employed. The stock solution is diluted for use in the proportion of 1 cc. of stain to 1 ounce of water. Staining dishes of 50 to 150 slides each are used; time of staining 1½ hours, after which slides are passed thru 2 changes of water, and air dried. Fresh rain water and city supply water are found to serve equally well for this work.

BUCK, J. B. Permanent aceto-carmine preparations. Science, **81**, 75. 1935.

An aceto-carmine preparation, considered worthy of preservation, is supported face down on 2 glass rods inside a Petri dish containing a mixture of equal parts of xylol, absolute alcohol, and glacial acetic acid. Previous scraping off of the excess paraffin or vaseline will hasten loosening of the cover glass. This being accomplished in 5 to 30 minutes, rinse the slide in same solution 5 minutes, drain and wipe clean. Pass thru 2 changes of xylol-absolute alcohol 1:1 (5 to 10 minutes), and xylol (10 to 15 minutes); mount in balsam. Smears of pollen mother cells remained unaltered after 10 months. A variant of the method by C. W. Metz and Elizabeth Gay is: soak off cover in equal parts of 95% alcohol, clove oil, glacial acetic acid; pass twice thru 95% alcohol up to ½ hour; absolute alcohol, 5 minutes; clove oil, 10 minutes; xylol, 5 minutes; balsam.

CRABB, E. D. Attaching refractory paraffine sections to the glass slip. Science, **80**, 530–1. 1934.

Fine cytological, as well as very difficult material, is securely fastened to the slip by this technic. Prepare the following two solutions: A) Collodion, considerably diluted with alcohol-ether 1:1; B) ⅛ amyl acetate, ⅞ Solution A. Spread sections and dry thoroly, flood with Solution B and place for 30 minutes face up on a level surface until nearly clear or free from paraffin. Blot vigorously by rolling a bottle over two or more layers of fine filter paper. Flood with Solution A and stand slip on end to drain and dry. Pass thru 70% alcohol (several minutes), rapidly thru 95% alcohol, into carboxylol or xylene (several hours); then thru 95, 70, 50% alcohol to water, and stain. After this and a cleansing in carboxylol, sections should be placed for several hours in xylol for removal of last traces of paraffin.

CUTULY, ELIZABETH AND CUTULY, E. Improvement of paraffin sections by immersion of embedded tissues in water. Science, **80**, 564–5. 1934.

Adrenal glands of rats are fixed in Wislocki's modification of Kohn's fluid, washed in formalin, passed thru graded alcohol up to 70%. The next step is treatment with H_2O_2 and alcohol for bleaching, and renders the material quite brittle. Fixation ends with clearing in a mixture of benzyl benzoate and methyl salicylate after absolute alcohol and before embedding. If the paraffin is shaved off from the block so that the tissue is just scratched and can soak up water, brittleness and electrification of sections upon cutting may be obviated; blocks thus prepared are immersed in water for 3 days to 2 weeks. Wet surfaces are dried and the blocks are dipped again in melted paraffin, avoiding stratification and bubbles. Similar results are obtained with intestine, stomach, liver, ovary, kidney, spleen, and tongue, variously fixed.

FORD, J. Modifications of Mann's and Giemsa's stains for sections of rabid material. J. Royal Micro. Soc., **54**, 263–4. 1934.

Demonstrations of Negri bodies in histological sections in the tropics fail to bring forth the proper color shade, red with Mann's and pink with Giemsa's method. In the case of Mann's stain the fault seems to be with the methyl blue (Grübler) used. Satisfactory results are obtained in the latter case by staining 3 hours at 37° C., rinsing and treating 5 seconds in straight formalin. After a rinse in water, continue as in Mann's methods; mount in neutral material, not in acid balsam.

HEUCKE, RUTH AND HENNEBERG, W. Volutinnachweis in den lebenden Hefezellen durch Neutralrot. Zentbl. Bakt., II Abt., **90**, 425–7. 1934.

Butter inoculated on a water agar tinted with 0.001 to 0.005% neutral red, resulted in a growth of red colonies of a non-red Entorula yeast. Within the colorless cytoplasm round red bodies lay beside uncolored ones. The latter stained orange red with a solution of Sudan, showing their fatty nature. The former ones are considered as volutin granules. Similar results were obtained with other yeasts, *Oospora lactis*, and *Thermobacterium bulgaricum*. The stain can be duplicated on the slide by mixing equal amounts of yeast suspension and dye solution. The resulting picture varies with the physiological condition of the cell. If dead, all of it takes on the stain at once. Live cells, instead, show different staining according to age, type, and source. A distinction is suggested between resting and active volutin. Even live yeast spores show a volutin granule.

HERZBERG, K. Viktoriablau zur Färbung von filtreierbarem Virus (Pocken-, Barizellen-, Ektromelia- und Kanarenvogelvirus). Zentbl. Bakt., I Abt., Orig., **131**, 358–66. 1934.

The writer claims to have stained filterable virus in the case of the following diseases: the canary disease of Kikuth, variola, varicella, ectromelia, and possibly in the case of herpes. The virus appears in the form of very minute granules stained blue to violet with Victoria blue. The dye employed by the author is "Victoria blue 4B hochkonzentriert" (Bayer standards, from Hollborn). A 3% solution of this dye in hot water is kept on a water bath at 60° for ½ hour, poured into a brown bottle and allowed to stand 2 weeks, filtering before use. The method of staining ordinarily employed is to apply this solution 5 to 20 minutes (according to depth of staining desired) to air-dried smears for 24 hours. They are then rinsed twice in distilled water for 30 seconds each time.

The canary virus is easily stained and ordinarily shows as violet granules within vacuoles in the histocytes. The smears are obtained from the oedematous portions of breast muscle, spread like a blood smear. The staining is equally simple in the case of ectromelia (Marchal's disease of mice); the smears are made by placing a slide against the swollen tissue around the source of infection. Material for variola is obtained by infecting a rabbit's eye, killing the animal 3 days later, and pressing a slide against the cornea. Staining may be as described above; or to secure more intense coloring, 0.3 cc. of a 10% aqueous solution of tartaric acid should be added to 10 cc. of the staining fluid. Varicella is more difficult to stain. The material is obtained by pressing a slide against a pustule in the first stage of eruption and smearing very thin with a cover glass. Staining should be for at least 20 minutes. To secure more intense staining the slides may be pre-treated in a sat. aqu. sol. of tartaric acid for 3 minutes at 60°C., rinsed twice (30 seconds each time) in distilled water, then 10 minutes at 37° in 3% Victoria blue to which 5% sat. aqu. tartaric acid has been added. (This is the method of G. Schwegmann.) In varicella the virus granules are very scarce; search should be made around the cells. In the case of herpes smears (made as in the case of variola) the air-dried smears should be placed in distilled water 10 minutes; then dried for an hour at 37°, and stained 30 minutes with Victoria blue. The granules are rare and feebly stained; author considers more evidence necessary to establish their nature.

Counterstaining has been undertaken in the case of the canary virus and variola. Smears are washed 10 minutes in distilled water, and dried 1 hour in the air, then stained 15 minutes in Domagk's Kernechtrot (5 g. aluminium sulfate disolved in 100 cc. hot water, filtered, 0.1 g. Kernechtrot (Hollborn) added, then 0.5 cc. of 10% acetic acid). They are rinsed twice (30 seconds each) in distilled water and transferred to a 1% solution of light green, (Hollborn), with 0.5 cc. per 100 cc. of 10% acetic acid. The light green staining is for 2 minutes in the case of the canary virus, 1 minute in the case of variola. The slides are rinsed twice for 5 seconds each in distilled water, and placed in 10% tartaric acid for 3 minutes. Two 5-second rinsings in distilled water are then given the canary virus slides, but all rinsing is omitted in the case of variola. They are then stained 20 seconds in Victoria blue (a solution freshly diluted to 0.1%, to 10 cc. of which 0.5 cc. of 10% tartaric acid is added). Rinsing in distilled water follows. By this procedure, the virus granules are amethyst violet, the cytoplasm of histocytes emerald green, nuclei salmon pink, and erythrocytes bright red, or covered with green or violet.

KRAJIAN, A. A. A rapid method for the demonstration of reticulum and collagen fibers in frozen sections. Arch. Path., **16**, 376–8. 1933.

Instead of 4 days, as with Perdrau's method, this technic requires about 1½ hours after the frozen sections have been cut. Altho a counterstain such as Van Gieson's solution can be used, it is not altogether necessary since the color differentiation between reticulum and collagen fibers is sharp.

Fix tissue in 1:10 formalin. Cut frozen sections 5 to 10 microns thick. Wash in 3 changes of distilled water. Soak 15 minutes in 10% NH_4OH at 60°C. Wash 3 times in distilled water. Soak 5 minutes in 0.3% $KMnO_4$. Wash a few seconds in distilled water. Decolorize in 1.5% oxalic acid until all the brown color is gone; treat sections separately and not longer than necessary. Wash 4 to 5 times in distilled water. Soak 1 hour in 5% $AgNO_3$ at 60°C. Wash twice in distilled water. Transfer for 15 minutes at 60°C. in the following ammoniacal $AgNO_3$ solution: to 8 cc. of 10%

$AgNO_3$ add 6 drops of 10% NaOH; make up a fresh 10% NH_4OH solution; add this to the precipitated silver solution drop by drop until almost entirely clear, or with only a few small particles of precipitate present; dilute to 28 cc. with distilled water. Wash quickly 3 times in distilled water. Transfer for 1 to 3 minutes to formalin (30 cc. formaldehyde solution and 70 cc. distilled water) at 60° C.

Wash in ample tap water and transfer sections onto slide. Dehydrate with a few drops of absolute alcohol and blot into position. Dehydrate further with two applications of absolute alcohol, blot, clear 3 minutes in 1:1 aniline oil and xylene, wash in xylene and mount.

The reticulum stains jet black; the collagen fibers deep brown. A toning process is appended in case photomicrographs are desired.

KRAJIAN, A. A. A modification of Dieterle's method for demonstrating *Spirocheta pallida* in single microscopic sections. Am. J. Syphilis, **17,** 127. 1933.

Quick demonstration of *Spirocheta pallida* in fresh necropsy matter would materially enhance a prompt and correct diagnosis of death. Dieterle's method is time-consuming. The following technic requires not over 1½ hours, is easier and gives clearer pictures.

Fix 5 mm. thick fresh tissue blocks in 10% formalin for 10 minutes at 70° C. Cut frozen sections (5 to 7 microns). [Transfer to 2% aqueous sodium cobalti nitrite for 5 minutes. Wash in two changes of distilled water.][1] Mordant 15 minutes at 70° C. in the following solution: uranium nitrate 1 g; 85% formic acid, 3 cc.; glycerin 5 cc.; acetone, 10 cc.; 95% alcohol, 10 cc. Wash a few seconds in distilled water. Develop 5 minutes in 10 cc. of the following solution to which, just prior to use, 1 drop of Mayer's glycerin-albumin is added: hydroquinone, 0.62 g.; sodium sulfite, 0.12 g.; acetone 5 cc.; 40% neutral formaldehyde, 5 cc.; pyridine, 5 cc.; saturated solution gum mastic in 95% alcohol, 5 cc.; distilled water, 30 cc. Wash a few seconds in distilled water. [Place sections in warm silver solution 15 to 25 seconds and wash in distilled water.][1] Transfer onto slides, dehydrate, clear and mount.

Control sections should always be run along with the unknown material. Keep all solutions in a dark and cool place.

KRAJIAN, A. A. A new elastic tissue stain, (including connective tissue, fibrin and amyloid). Arch. Path., **18,** 378–80. 1934.

For the demonstration of elastic tissue, connective tissue and fibrin, fix tissue in 10% formalin 24 hours or longer. Cut by freezing. Wash in tap water. Treat sections in 2% aluminum chloride 5 to 10 minutes. Wash. Stain 10 minutes in a mixture of 8 cc. of a 4% Congo red in 5% aqueous sodium citrate with 2 cc. glycerin, C. P. Wash in tap water. Transfer sections to 1% aqueous KI solution for 10 seconds and agitate. Wash in tap water. Stain 5 to 10 minutes in the following fluid: anilin blue, 1.5 g.; orange G, 2.5 g.; resorcinol, 3 g.; phosphomolybdic acid, 1 g.; distilled water, 100 cc. Wash thoroly in plenty of tap water; float sections onto slides and blot with filter paper. Dehydrate 2 minutes in absolute alcohol, changing alcohol twice (dropping bottle method). Clear 2 minutes in oil origanum, then xylene. Mount in damar.

Elastic fibers stain bright red, fibrin and connective tissue dark blue. Sections from paraffin embedding require longer staining (30 minutes in each of the two solutions). A modification of above Congo red stain is proposed for staining amyloid. Stain loose frozen sections in Harris' hematoxylin 15 seconds and wash in tap water until sections are light blue. Transfer to 4% aqueous Congo red with glycerin 15 minutes and wash in tap water. Differentiate in 1% aqueous KI and wash in tap water. Transfer sections to slide and dehydrate as given above. Clear in xylene 2 to 5 minutes. Mount. Amyloid material stains red.

OKINO, K. Metallimprägnationsversuche an den Erythrozyten. I. Imprägnierung der Erythrozyten verschiedener Tierarten. Folia Anatomica Japonica, **12,** 373–81. 1934.

Impregnation by means of Ag-ions allows a classification of the blood of various animals which checks quite well with that based on the electric charge of the different erythrocytes.

[1]The steps given in the brackets are not in the published paper, but were added by the author in a personal communication.

Fresh blood smears from several species are made side by side on the same glass slip, air dried, and treated as follows: 1) Soak 10 to 30 minutes in 100 cc. of a freshly prepared 2% $AgNO_3$ solution plus 1 to 2 drops ammonia. 2) Rinse in distilled water. 3) Reduce 1 to 3 minutes in the following freshly made up and filtered reagent: 1% hydroquinone, 90 cc.; formalin, 10 cc.; 2% $AgNO_3$, about 25 drops. 4) Rinse in distilled water. 5) Dehydrate and mount.

OLIVER, W. W. Staining of the processes (flagella) of human erythrocytes. J. Inf. Dis., **55**, 266–70. 1934.

Processes of the flagella type have so far been observed on erythrocytes only in fresh blood preparations. The following is a method of staining recommended for fixed smears. To 2 to 3 cc. of sterile Ringer's solution in a sterile test tube add 1 mg. of hirudin or some such anticoagulant. Draw up into a Pasteur pipet about 0.5 cc. of this liquid and mix it with a drop of fresh normal human blood. Transfer this blood to the test tube and incubate for 40 to 50 minutes at 37° C. Tilt the glass slips at an angle of about 40° and let a small drop of the blood mixture run down from the upper edge. Move carefully to a horizontal position and air dry. Soak film with the following mordant: 10% tannic acid, 50 cc.; saturated aqueous $FeSO_4$, 25 cc.; saturated alcoholic basic fuchsin, 5 cc. Make up fresh each time and filter at least twice. After covering the slide, warm slightly and let mordant act 20 minutes. Wash in running water and blot dry. Stain 20 minutes with a fresh Ziehl-Neelsen carbol fuchsin (containing 1% fuchsin); wash thoroly in tap water, blot dry.

Erythrocytes stain light pink, dark red or occasionally purplish black, in most cases irregularly. If there are endoplastic bodies they stain a deeper hue than the surrounding cytoplasm. The extroflecting processes are light pink, like the cell membrane.

PARKER, B. W. An electrically-heated slide-ringing device. Science, **80**, 456–7. 1934.

Permanent slides of materials mounted in non-hardening media, such as lactophenol, are usually secured by fixing the coverslip with a suitable cement. A satisfactory one is Noyer's, consisting of 80% rosin and 20% anhydrous lanolin. An electrical heating appliance is described in detail which insures uniform and satisfactory temperature of the wire coil with which proper application of the cementing medium may be attained.

PATAY, R. Une nouvelle technique de coloration trichromique pour l'histologie. Bull. d'Histol. Appl., **11**, 408–9. 1934.

The method developed for the histological study of a coleopter is suitable for sections of arthropod, shell fish, and mammalian tissues. All usual fixing agents can be used except osmic acid. Embed in paraffin, cut and run down to water. Stain 5 minutes in Masson's hemalum (boil 100 cc. of saturated potassium alum, dissolve in it 2 g. hematein, cool, filter, and add enough acetic acid to turn the color to a definite red). Wash a few minutes in running water. Stain 2 minutes in 1% xylidin ponceau in distilled water. Wash rapidly and treat 2 minutes with 1% phosphomolybdic acid in distilled water. Wash again carefully and stain briefly (30 seconds) with a 0.5% light green solution in 90% alcohol. (No information given as to source of stains.) Dehydrate and mount. Chitin and connective tissue stain green; cytoplasm of epithelium cells, orange-brown; nuclei, violet-brown; muscle, yellow-orange; blood, golden yellow; nerve tissue, gray-brown.

REISINGER, E. Neues zur vitalen Nervenfärbung. Verh. deutsch. Zool. Ges., **35**, 155–60, 1933. (Abs. in Zts. Wis. Mikr., **50**, 368. 1934.)

Live Turbellariae are treated with carbonate, 1 part of concentrated aqueous lithium carbonate to 200 to 600 cc. of the culture fluid, for 15 minutes to several hours, depending on the temperature and concentration. They are next rinsed in water and transferred for 2 to 8 hours to alizarin in diffuse light. Dissolve 1 to 3 g. of sodium lysalbinate (Schuchardt in Görlitz) in 100 cc. water, bring to boil and add a small amount of alizarin from the tip of a scalpel, cool quickly and filter.

Successful preparations should reveal a dark violet blue peripheral nervous system. Alizarin precipitates on nerve end surfaces.

RUYTER, J. H. Une méthode d'entourage pour les préparations. Bull. d'Histol. Appl., **11**, 410. 1934.

The following technic is offered as an improvement over the use of Noyer's lake for sealing preparations mounted in levulose-gelatin or glycerin.

To 100 cc. of liquefied 20% gelatin in saturated aqueous solution of thyme add 10 cc. of a 5% $K_2Cr_2O_7$ solution and mix. Preserve in the dark. Whenever needed, seal preparations with it by using a small brush; let it dry at room temperature exposed to light.

SPARK, C. A simple differential stain for the human hypophysis. J. Lab. & Clin. Med., **20**, 508–9. 1935.

Fix 48 hours, preferably in Orth's liquid (Müller's fluid, 9 parts; formol, 1 part) to minimize brittleness of tissue. Wash 24 hours in running water. Run paraffin sections down to water and stain as follows: 0.25% aqueous anilin blue, 60 to 90 seconds. Wash 30 seconds in tap water. Immerse in Mayer's hematoxylin, 10 minutes, and wash again in tap water for 2 to 3 minutes. Immerse in Van Gieson's stain, 60 to 90 seconds. Wash in tap water 1 minute. Transfer to 95% alcohol 1 minute, dehydrate 2 to 3 minutes in absolute alcohol. Clear and mount. Cellular elements of human hypophysis are thus clearly defined: nuclear chromatin stains purplish brown; basophile cells and beta granules, dark blue; alpha granules, olive green; cytoplasm of chromophobe cells, light grayish blue; erythrocytes, bright yellow.

TOMITA, T., FUJITA, T. and IZUMI, M. Weitere Mitteilung über die feinere Struktur der Erythrozyten von Säugetieren mit der neuen Silberimprägnationsmethode mit besonderer Berücksichtigung von der Existenz der feineren Struktur in den Menschen-Erythrozyten. Folia Anatomica Japonica, **12**, 445–56. 1934.

A new method of silver impregnation for the study of the fine structure of erythrocytes, a development of the Fujita technic, is applied in the following manner: Suspend the blood in 0.8% saline and centrifuge; wash cells repeatedly with saline. Fix 30 seconds by adding Champy's reagent; centrifuge; wash 3 to 4 times with distilled water. Impregnate by adding a 1% gelatin-hydroquinone reagent (1 part 2% Nelson's photographic gelatin No. 2 plus 1 part 2% hydroquinone) as a reducing agent and holding 4 minutes at 65° C.; then rinse once with distilled water and treat for 2 minutes at 65° C. with a 1% gelatin-silver impregnating agent (1 part 2% Nelson's photographic gelatin No. 2 plus 1 part 2% silver nitrate). This impregnation step might be followed by a further gold treatment. Dehydrate in the alcohol series, xylol, xylol-balsam, and mount.

v. VOLKMANN, R. and STRAUSS, F. Ein Ersatz für die Hornowskysche Kombination zur Darstellung von Elastin, Muskulatur und Kollagen auf der Basis der Azanmethode. Zts. Wis. Mikr., **51**, 244–9. 1934.

A sharp and lasting method of differentiation between elastic, muscular and collagenous tissues is greatly needed, especially for purposes of teaching. The following is based on Heidenhain's *Azan* technic, in turn a development of Mallory's method. Nearly all common fixatives are satisfactory but are best when containing $HgCl_2$. 1) Dehydrate in 95% alcohol. 2) Stain 1 to 12 hours in resorcin-gentian violet elastin. (The stain is prepared like Mallory's resorcin-fuchsin, minus the acid.) It gives a deep blue color and does not require any special differentiation afterward. 3) Rinse 1 to 2 minutes in 70 to 95% alcohol (in exceptional cases in acid alcohol). 4) Water. 5) Azo carmin G nuclear and muscular stain (strong acid solution) 25 to 30 minutes at room temperature. 6) Rinse, differentiate in anilin alcohol. 7) Five percent phosphotungstic acid until collagen appears completely decolorized. If necessary, rinse again in *water-free* anilin alcohol. 8) Water. 9) One per cent naphthol green in 1 to 2% acetic acid (occasionally 0.06% alizarin-viridin in water), as a collagen stain, 25 minutes to several hours. 10) Water. 11) Dehydration beginning with 80% alcohol. 12) Creosote or carbolxylol; xylol.

Elastic fibers are stained deep blue; chromatin, plasma and muscle elements, red; collagenous tissue, green.

This technic has been used in histology class work for 2 years.

WALLART, J. and HOUETTE, C. Une coloration trichromique rapid à l'hématoxyline, la fuchsine acide et le jaune solide. Bull. d'Histol. Appl. **11**, 404–7. 1934.

A method is proposed for obtaining trichrome staining, similar to Masson's, after practically any fixative, altho Bouin's and Susa's seem to offer the most brilliant results. The technic is as follows: Make up the staining solution by mixing equal volumes of: a) 1% acid fuchsin (Krall or Hollborn) plus 1% of acetic acid; b) 3% fast yellow G or GG (Ciba) plus 1% of acetic acid; c) 1% phosphomolybdic acid. After the sections are brought down to tap water, stain with a few drops of Weigert's iron hematoxylin (3 minutes when fresh, ½ to 1 minute if more than 2 days old). Rinse in tap water, differentiate quickly with 70% alcohol plus 0.5% of HCl and wash 1 to 2 minutes in tap water. Stain 5 minutes with the triple stain mixture. Rinse in tap water and treat 5 minutes in 1% acetic acid in distilled water. Wipe as much of the slide as possible with a dry cloth and treat 30 seconds by drops with 1% acetic acid in absolute alcohol. Complete dehydration in absolute alcohol. Clear and mount in salycilic balsam. Chromatin stains black or dark violet; cytoplasm red with brilliant granulations; collagenous fibers all shades of yellow; and elastin, pink.

ZACH, C. Colorazione macroscopica di colture del bacillo tubercolare su terreno Petragnani a scopo fotografico. Bol. Ist. Sierotera. Milan, **13**, 297. 1934.

Two methods are given for staining macroscopically tubercle bacillus cultures growing on slants of Petragnani's agar for photographic purposes. 1) A modified Haase's technic suitable for scattered growth: fill the culture tube with Giemsa's solution and hold it 12 hours at 37°C; replace the solution with 5% acetic acid, changing several times; replace the acid with 10% $Na_2S_2O_3$ at 37°C, changing repeatedly until the medium appears slightly purplish and the colonies deep blue. 2) Suitable for richer and thicker growth: fill the tube with a 10% solution of Ziehl's carbol-fuchsin and hold 12 hours at 37° C. The light yellow colonies stand out sharply on the deep red of the background, and are readily photographed.

ZIRKLE, C. Butyl alcohol and cytological technique. Science, **80**, 481. 1934.

On minor uses of *n*-butyl alcohol: 1) A mixture of ⅔ ethyl and ⅓ butyl alcohol can keep both water and xylol in solution; 1 part water, 1 part xylene, 1 part *n*-butyl alcohol, 2 parts ethyl alcohol, yields a clear solution useful in cleaning slides of most mounting and sealing materials. If dilute ammonia is substituted for water, and carbon tetrachloride for xylene, a still more potent cleanser is obtained. 2) A few drops of *n*-butyl alcohol added to the absolute alcohol and xylene jars prevent cloudiness due to water contamination. Example of a series: xylene; xylene 95%, and *n*-butyl alcohol 5%; absolute ethyl alcohol 90% and *n*-butyl alcohol 10%; absolute ethyl alcohol, etc.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stain approved since the last one listed in the January number of this journal.

STAINS CERTIFIED DECEMBER 1, 1934 TO FEBRUARY 28, 1935*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methyl orange	CM 4	94%	As histological counterstain	Dec. 14, 1934
Phloxine	NPh 7	86%	For histological staining	Jan. 15, 1935
Methylene Azure A	NAz 5	90%	As histological stain and as constituent of blood stains	Jan. 21, 1935
Basic fuchsin	NF 27	85%	As bacteriological and histological stain; also in Endo medium	Jan. 22, 1935
Methylene blue	NA 9	88%	As histological and bacteriological stain; as constituent of blood stains	Jan. 25, 1935
Janus green B	NJ 4	64%	As vital stain for blood	Jan. 25, 1935
Erythrosin	NEr 3	87%	As histological stain	Jan. 25, 1935
Gentian violet	CB 11	88%	As histological, cytological and bacteriological stain	Jan. 25, 1935
Eosin Y	CE 7	89%	As histological stain and as constituent of blood stains	Jan. 25, 1935
Brilliant cresyl blue	NV 14	62%	As vital stain blood	Feb. 14, 1935

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.